Changes in Composition of Culturable Bacteria Community in the Gut of the Formosan Subterranean Termite Depending on Rearing Conditions of the Host

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ABSTRACT The hindgut of feeding termites that feed on wood and litter contains a diverse population of bacteria and protists that contribute to the carbon, nitrogen, and energy requirements of the termite. For understanding the ecological balance in the termite gut, detailed knowledge about the composition of the microbial gut flora is imperative, i.e., the numbers and relative proportions of the microbial taxa and the variability in the microbial composition among different termite colonies and living conditions of termites should be described. Therefore, we isolated and enumerated eight bacterial morphotypes from the gut of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki. Five morphotypes (three isolates of lactic acid bacteria, isolates of the family Enterobacteriaceae and isolates belonging to the genus *Dysgonomonas*) were found frequently in all termite colonies. Three additional morphotypes were found sporadically and were considered to be transient flora. We compared the proportions of the three lactic acid bacteria isolates and the Enterobacteriaceae among three different termite colonies. Furthermore, we investigated the shift in proportions of these four major morphotypes depending on whether bacteria were isolated from freshly collected termites or from termites reared in the laboratory under seminatural conditions (in arenas on wood) or artificial conditions (in petri dishes on filter paper). Differences in the culturable microbial composition were not significant among termite colonies, or between field-collected termites and termites reared under seminatural conditions in the laboratory. However, we found significant shifts in the microbial composition between field-collected termites and termites reared on filter paper.

KEY WORDS *Coptotermes*, Isoptera, Rhinotermitidae, insect gut, gut symbionts

Wood and litter-feeding termites (Isoptera) are of global economic and ecological importance as decomposers of lignocellulose matter (Kambhampati and Eggleton 2000). To be able to digest lignocellulose efficiently and use lignocellulose as sole source of nutrition, termites harbor a morphologically and biochemically diverse microbial flora in their intestines. Densities of microbial populations in termite intestines are as high as 10^{12} per ml gut fluid, and thus similar to other herbivorous and detrivorous invertebrates and even vertebrates (Bignell 2000). In addition to protists and fungi, there is a significant community of prokaryotes from the domains of Archaea and Eubacteria with densities of 10^{9} to 10^{11} per ml gut fluid (Breznak 2000).

The majority of microbes in the termite gut are yet uncultured and unidentified, and their role in termite nutrition is not well understood. Studies eradicating the termites' intestinal flora through antibiotics (Eutick et al. 1978b, Mauldin et al. 1978) or oxygen (Veivers et al. 1982) indicate that symbionts vital for the survival of the termite host species are within the microbial community. Their roles include nitrogen fixation, acetogenesis, cellulose degradation, maintenance of pH and redox potential in the gut, as well as preventing foreign microbes from invading (Veivers et al. 1982, Bauer et al. 2000, Breznak 2000). Additionally, the bacteria composition might influence nestmate recognition (Matsuura 2001).

Because of the vital role of the gut flora for the termite host's survival, it could be assumed that selective pressures ensure a comparable microbial community among termites of the same species, with the most important microbe groups always present (Schmitt-Wagner et al. 2003, Hongoh et al. 2005, Yang et al. 2005). However, not all of the gut inhabiting microbes are necessarily symbionts sensu strictu (providing vital advantages to the termite host); some microorganisms might be transient and subject to environmental factors, such as nutrition, which could lead to a considerable variation of the composition of gut flora within the same termite species.

Because termites are social insects living in colonies, the variation of the gut flora within a species can vary at different levels of social organization. Between ter-

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mites within the same colony variation is supposed to be low (Minkley et al. 2005), because colony members live under the same conditions and commonly use the same nutrition source (Matsuura 2001). Additionally, the isolation of bacterial populations in the guts of individual termites is constantly counteracted by the exchange of gut fluids containing microbes via proctodeal and stomodeal trophallaxis between colony mates and by the obligatory refaunation after molting (McMahan 1969, Thorne 1997). However, termites of different colonies usually do not interact with each other and therefore do not exchange microbial flora. In addition, geographically separated termite colonies might be subjected to different ecological conditions and use different nutrition sources. Different chemical components in the food might favor different microbial groups becoming predominant (Mannesmann 1972).

The presumed variation in the composition of the microbial gut flora might explain why studies culturing, identifying and enumerating microbial taxa differ from each other even when focusing on the same host species (Mannesmann and Piechowski 1989, Taguchi et al. 1993, Husseneder et al. 2005, König et al. 2006). Attempts to track the factors causing variation in the microbial gut communities within termite species by reviewing the literature are hindered by the fact that most authors did not clearly state whether the investigated termites have been collected from the same colony, nor whether the termites have been freshly collected from the field or have been laboratory reared. In some studies, termites were laboratory reared and fed either on wood or filter paper (Wenzel et al. 2002). However, it has not yet been established whether the bacterial composition changes with rearing conditions and nutrition.

To put findings on microbial variability within the same termite species into perspective, we investigated the variability of the composition of the culturable gut flora of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). This termite is an invasive pest species in the United States, inflicting extensive economical damage in Hawaii and the southeastern states. Shinzato et al. 2005 used culture independent methods (16S rRNA gene sequencing) to describe the bacterial species composition in the gut of Japanese *C. formosanus* as a first step toward the understanding of the ecology of the gut of this termite. These authors found 49 phylotypes from 10 bacterial phyla, including 39 novel phylotypes but did not investigate potential variations between termite colonies. In our study, we described the major culturable bacterial morphotypes in the gut of the Formosan subterranean termite and investigated the variability of the microbial composition between termite colonies and how the relative proportions of predominant morphotypes change depending on rearing conditions and diet of the termite host.

Materials and Methods

Termite Collection and Rearing. Workers and soldiers of *C. formosanus* were collected from traps made from Douglas fir, *Pseudotsuga menziesii* (Mirbel) Franco, in 2000 and 2001 from three collection sites, located on the campus of the University of Hawaii at Manoa (Gilmore [G], Miller [M], and Publication [P]). Physical distance between collection sites ranged from 120 to 400 m (for a detailed map, see Husseneder and Grace 2001). Previous studies, using molecular genetic methods, established that termites from these three collection sites belong to three independent colonies (Husseneder and Grace 2001). From each colony, >250 workers and at least 20 soldiers were collected. The composition of the culturable gut flora of termites from each of the three colonies was assessed under three different rearing conditions. 1) To investigate the natural gut flora, five workers of each colony were dissected within a few hours of removal from their colony in the field. 2) To investigate the gut flora under seminatural conditions in the laboratory, we established a laboratory colony composed of 100 workers and 10 soldiers from each field colony in arenas (25 by 25 cm) containing 100 g of sand with 25 ml of distilled water added. The worker-to-soldier ratio mimics natural conditions (Haverty 1977). Termites were maintained on a diet of Douglas fir wafers, and five workers of each colony were dissected after seven days to isolate their gut flora. 3) To investigate the changes of gut flora under artificial rearing conditions, we kept 100 workers and 10 soldiers in petri dish (90 mm diameter) arenas on a diet of Whatman no. 3 (70 mm diameter) filter paper moistened with distilled water. The paper was changed every 3 d and water was added when needed. After 7 d, the guts of five workers from each colony were dissected to isolate and culture the bacteria.

Isolation of Gut Bacteria. Five workers from each of the three colonies and each of the three rearing conditions were anesthetized by chilling on ice, the mouth and anus of the termites were sealed with paraffin, and the surface was sterilized with 70% alcohol. Guts were removed with sterile forceps. The whole hindgut portion was separated from the midgut and homogenized in 1 ml of sterile distilled water with a sterile glass rod in an autoclaved Eppendorf tube and then vortexed for ≈4 min with sterile glass beads. Isolation and homogenization were performed under aerobic conditions. Ten-fold serial dilutions were made from homogenates with distilled H_2O and plated out in triplicates on Todd-Hewitt agar (BD Biosciences, Cockeysville, MD). Plates were incubated for 3 d at 30°C under anaerobic conditions using a GasPak jar containing a GasPak H_2/CO_2 envelope (BD Biosciences).

Enumeration and Characterization of the Bacterial Isolates. Gram stains, colony, and cell morphology were used initially to assort bacterial isolates into eight morphological types (morphotypes). The absolute number of bacterial colony-forming units (CFUs) of the eight morphotypes in each termite gut was deter-

mined for each termite colony and each rearing group separately. Enumeration was performed by averaging the number of isolates on three countable dilution plates. Proportions of the dominant morphotypes were calculated and compared between termite colonies and rearing groups. Bacterial colonies of each morphotype were recovered and isolated on Todd-Hewitt plates for further characterization using a series of classical biochemical and physiological tests.

Aerotolerance of isolates was assayed by plating recovered organisms onto Todd-Hewitt agar and monitoring growth at 30°C for 1 wk under normal atmospheric conditions. Aerotolerant organisms were tested for catalase and cytochrome oxidase. Fermentation of carbohydrates (α-CH_3-D-gluconate, cellobiose, dulcitol glucose, glycerol, inositol, lactose, maltose, mannitol, melibiose, melizitose, raffinose, rhamnose, salicin, trehalose, and xylose) was assayed using a 1% concentration in phenol red broth base with mineral oil overlays and incubated for 1 wk. Methyl red and Voges-Proskauer tests were assayed using MR-VP broth in tubes (with mineral oil overlays); test reagents were added when the content of the tubes showed turbidity. Hemolysis was assayed using 5% sheep blood agar plates grown anaerobically for 3 d. Stab cultures in Bile Esculin Agar were incubated for 1 wk to determine sensitivity to bile and ability to use esculin. Triple Sugar Iron agar tubes were used to further characterize members of the Enterobacteriaceae. The API 20E system (bioMerieux, Inc., Durham, NC) test strips were used to identify members of the family Enterobacteriaceae to the species level if possible.

Motility of bacteria was tested using two basic tests. First, a hanging drop suspension was prepared from a culture of the organism grown in Todd-Hewitt broth and the microbe was assayed for motility by using phase contrast microscopy. Second, a tube of thioglycolate broth was inoculated using a wire needle and monitored for 7 d for the presence of motility. Thioglycolate broth contains 0.5% agar so that motility can be assayed through examining the tube for microbial growth that extends beyond the point of the initial inoculation.

Scanning electron microscopy was performed by the Socolofsky Microscopy Facility at Louisiana State University. Cultured bacteria were fixed with 2% glutaraldehyde and 1% formaldehyde, postfixed with 2% osmium tetroxide, rinsed, applied to graphite-coated specimen mounts, air-dried, sputter coated, and imaged with a Cambridge 260 scanning electron microscope.

Statistics. We tested how two factors, i.e., colony membership (colonies M, G, P) and rearing group (field colonies, laboratory reared in arena on wood, laboratory reared in petri dish on filter paper) influence the proportions of bacterial morphotypes in the guts of termites. Overall, eight bacterial morphotypes were found. However, the first data exploration showed that the statistical power was diluted because of high variance in four morphotypes, which occurred infrequently ("coccobacillary," "oval," and *Cellulomonas* sp.) or were obviously dependent on another morphotype (*Dysgonomonas* spp. grew mainly as satellite bacteria around Enterobacteriaceae colonies). Therefore, we included into the statistical analysis only the major isolates that were consistently found and grew independently (isolates 1–3, and Enterobacteriaceae).

Absolute numbers of bacterial counts were converted into percentage proportions and the percentage raw data were subjected to arc sine transformation (Zar 1996). The effects of the two factors, termite colony and termite rearing on the dependent variables, i.e., proportions of the four major morphotypes, were analyzed using the multivariate version of the General Linear model as implemented in the software package SPSS 11.5 (SPSS Inc., Chicago, IL.). Additionally, post hoc tests (Tamhane's T2) for multiple comparisons were performed for each factor and each variable separately.

16S rRNA Gene Sequencing. Bacterial DNA was extracted using phenol-chloroform extraction and the protocols of Ausuebel et al. (1992). The nearly complete 16S rRNA gene was amplified in two steps using the primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 926r (5′-CCGTCAATTCCTTTAAGTTT-3′) and 533f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). Polymerase chain reaction (PCR) reagents and conditions are described in Hugenholtz et al. (1998). Sequencing of the 16S rRNA gene was preformed by the BIOCORE facility at University of Hawaii at Manoa and by GeneLab at Louisiana State University by using Applied Biosystems (Foster City, CA) sequencers. Taxonomic identification of bacteria from 16S rRNA gene sequences was obtained by using the online resource BLAST (Altschul et al. 1997), maintained by the National Center for Biotechnology Information (Bethesda, MD).

Results

Bacterial Groups Isolated from the Gut. Classical bacteriological identification techniques revealed eight groups of morphotypes isolated and cultured from the guts of *C. formosanus* from three different field colonies in Hawaii. Of the five major morphotypes, which were present in all termite colonies in considerable numbers, three strains of lactic acid bacteria could not be identified to the genus or species level by classical methods (isolates 1–3). The fourth morphotype comprised several species of the Enterobacteriaceae. The fifth morphotype consisted of *Dysgonomonas* species, which grew mainly as satellite bacteria around Enterobacteriaceae colonies. Three additional morphotypes ("coccobacillary," "oval," and *Cellulomonas* sp.) were found infrequently and were thus considered transient flora. The eight morphotypes were characterized by their morphology and biochemistry (Fig. 1).

Isolate 1 was a gram-positive irregular rod, which grew under anaerobic conditions at 25 and 35°C but not at 42°C. Bacteria cells occurred singly or in pairs

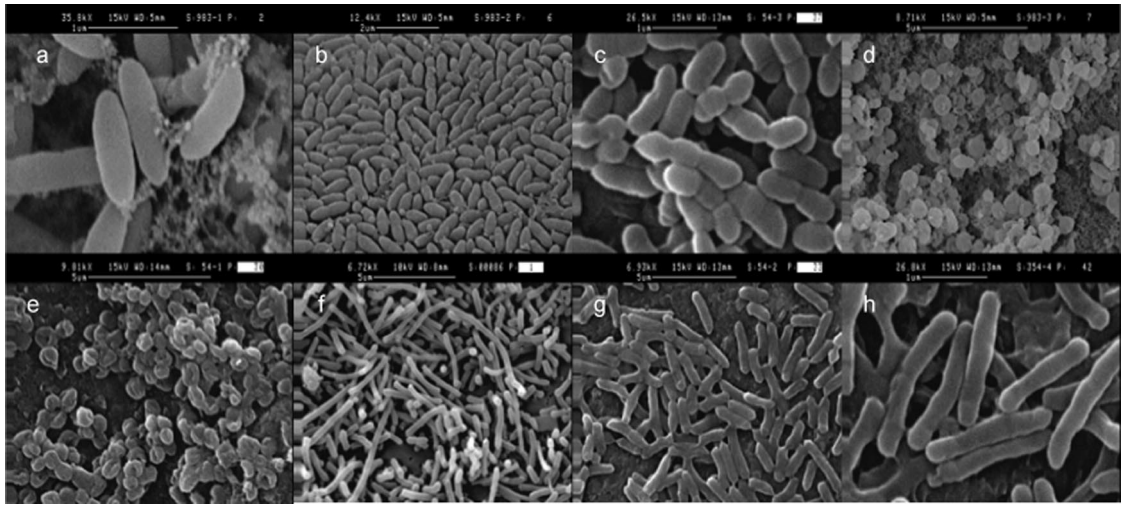

Fig. 1. Scanning electron micrographs of the eight bacterial morphotypes isolated from the gut of *C. formosanus*. Five novel species of lactic acid bacteria (a, isolate 1; b, isolate 2; c, isolate 3; d, oval; e, coccobacillary), a novel *Dysgonomonas* species (f), an unknown member of the Enterobacteriaceae (g), and a novel *Cellulomonas* species (h).

and seemed tapered and slightly curved and were not motile. Colonies were convex and cream colored with irregular margins. Isolate 1 fermented glucose, fructose, lactose, maltose, melibiose, rhamnose, salicin, trehalose, and xylose with lactic acid as the main fermentation product. Thus, isolate 1 is affiliated with the Lactobacillales (lactic acid bacteria). Strains were catalase negative; they typically reacted positive for bile esculin and showed weak beta hemolysis on blood agar. The 16S rRNA gene sequence (GenBank accession no. AY533171) matched closest with that of an uncultured bacterium from *C. formosanus* from Japan (99% similarity, AB062833, Shinzato et al. 2005).

Isolate 2 was a gram-positive coccobacillary that nevertheless showed a considerable variability in its gram characteristics depending on strain and growth conditions. Cells occurred singly or in pairs and were often capsulated. Colonies were convex and cream colored with round to irregular margins. Like isolate 1, this organism was nonmotile, anaerobic and fermentative using a variety of sugars, with the main fermentation product of lactic acid. Isolate 2 reacted positive for the methyl red test. The 16S rRNA gene sequence of isolate 2 (accession no. AY533172) matched closest to that of another uncultured bacterium from Japanese *C. formosanus* (98% similarity AB062811, Shinzato et al. 2005).

Isolate 3 was variable in its morphology and size, ranging from gram-positive irregular rods to coccoids. The cells occurred singly or in chains, seemed often swollen and irregular, and were nonmotile. Colonies were convex with regular margins and ranged from opaque to cream colored. Like isolates 1 and 2, this bacterium was anaerobic and lactic acid producing. However, isolate 3 did not ferment any of the standard carbohydrates. This biochemical inertia impeded detailed physiological and biochemical characterization. Comparison of the 16S rDNA sequence of isolate 3 (AY533174) to sequences in public databases placed this bacterium in the order Lactobacillales. However, the lack of any match in GenBank with a sequence similarity closer than 95% suggests that isolate 3 is a novel species in this order.

Three members of the family Enterobacteriacea were isolated from the termite gut in this study. All strains were gram-positive facultative anaerobe motile rods. Strains identified as *Enterobacter cloacae* using API20E test strips exhibited some variation. For example, some isolates fermented lactose and others did not, some isolates were methyl red positive, and others were Voges-Proskauer positive. Two other Enterobacteriaceae strains could not be identified to the species level.

"Satellite bacteria" frequently grew in clusters around the colonies of the Enterobacteriaceae. Typically, the cells were gram-negative, pleiomorphic, nonmotile rods of variable length. Some strains tolerated oxygen and were able to grow aerobically, whereas others did not. These satellite bacteria were fastidious and grew very slowly with visible colonies occurring after 5 d. Colonies were small ("pinhead size") raised and opaque with round margins. Sequencing the 16S rRNA gene region of two different strains identified these satellite bacteria as two possibly novel species of the genus *Dysgonomonas* (GenBank accession nos. AY 571962, AY 581890).

In addition to the five major morphotypes, we sporadically found three minor morphotypes. The first was a coccobacillary lactic acid bacterium, which resembled isolate 2 in its morphology (Fig. 1). However, unlike isolate 2, the coccobacillaries fermented only glucose. The second minor isolate (oval) was a gram-positive nonmotile coccus that grew well under aerobic conditions. Cells occurred singly or in chains and were oval shaped. Colonies were convex and cream colored with irregular margins. Both, coccobacillary

Table 1. Culture-dependent enumeration of the eight bacterial morphotypes isolated from *C. formosanus* guts

Morphotype	Rearing	Colony M			Colony G			Colony P			All colonies		
		Avg.	SD	%	Avg.	SD	%	Avg.	SD	%	Avg.	SD	%
Isolate 1	Field	147	99	67	12	7	7	16	2	28	58	78	40
Isolate 2		71	21	33	107	27	65	20	5	34	66	32	45
Isolate 3		0	0	0	22	22	13	2	2	3	8	16	5
Enterobacteriaceae		0	0	0	1	1	1	5	4	9	2	6	2
Dysgonomonas spp.		0	0	0	5	4	3	14	10	25	6	8	4
Coccobacillary		0	0	0	0	0	0	0	0	0	0	0	0
Oval		0	0	0	0	0	0	0	0	0	0	0	0
Cellulomonas sp.		0	0	0	17	17	10	0	0	0	6	13	4
Isolate 1	Wood	8	4	8	8	2	6	27	16	26	14	13	12
Isolate 2		30	17	32	30	11	21	48	14	46	36	18	32
Isolate 3		25	13	26	30	9	21	4	2	3	20	13	17
Enterobacteriaceae		4	2	4	4	3	3	4	1	3	4	3	4
Dysgonomonas spp.		17	33	18	72	42	49	22	12	21	37	35	32
Coccobacillary		9	8	10	0	0	0	0	0	0	3	6	3
Oval		1	1	1	0	0	0	0	0	0	0	1	0
Cellulomonas sp.		0	0	0	0	0	0	0	0	0	0	0	0
Isolate 1	Paper	8	5	9	20	13	6	37	21	24	22	19	12
Isolate 2		1	1	1	27	18	9	46	28	30	25	26	13
Isolate 3		20	9	24	142	78	45	22	11	14	61	64	33
Enterobacteriaceae		13	5	16	3	2	1	16	6	10	10	6	6
Dysgonomonas spp.		39	12	48	111	98	35	29	18	18	60	72	32
Coccobacillary		1	1	1	0	0	0	0	0	0	0	1	0
Oval		0	0	0	0	0	0	6	4	4	2	3	1
Cellulomonas sp.		0	0	0	14	14	4	0	0	0	5	11	3

Numbers refer to colony-forming units (CFUs) of bacteria. Bacteria were isolated from three termite field colonies (M, G. P) and termites of the same colonies reared in the laboratory under seminatural conditions (wood-fed) and artificial conditions (filterpaper fed). The averages of CFUs per colony are based on five individuals.

and oval morphotypes had lactic acid as their major fermentation product (lactic acid bacteria). The third minor isolate was not very common. This organism was a gram-positive nonmotile short rod, which typically occurred in chains. Cells sometimes seemed filiform in older cultures. Colonies were convex with regular margins and frequently acquired a yellow pigment in older cultures. This organism was facultative anaerobe and fermentative. A partial 16S rRNA gene sequence (unpublished) together with the biochemical characteristics placed this bacterium into the genus *Cellulomonas.*

Enumeration of the Bacterial Isolates from Termite Field Colonies and Laboratory-Reared Termites. Table 1 shows the culture dependant enumeration of the eight bacterial morphotypes based on CFUs cultured from termites from field colonies and termites which were reared in the laboratory under seminatural conditions (wood-fed) and artificial conditions (filter paper-fed). Isolate 1 and 2 were present in all investigated termite workers from all three field colonies. Although isolates 1 and 2 were predominant in field colonies combining 62–100% of the cultured flora, isolate 3 and the Enterobacteriaceae were present only in colony G and P (Table 1).

The dominant proportions changed when considering laboratory-reared colonies (Table 1). Under seminatural rearing conditions (wood-fed termites) the counts of isolates 1 and 2 were diminished. Under this rearing condition, isolate 3 and Enterobacteriaceae were found in all three colonies and became more frequent compared with their proportions in field-collected termites. Isolate 3 and Enterobacteriaceae became even more dominant in laboratory-reared termite colonies that were fed filter paper.

Besides those four morphotypes, *Dysgonomonas* spp. were detected in varying numbers as satellite colonies around Enterobacteriaceae colonies. *Cellulomonas* sp. was found only in a few termite workers of colony G (86×10^3 CFUs per whole gut in one field caught individual and 71×10^3 CFUs per whole gut in one laboratory-reared filter paper-fed individual). Oval-shaped bacteria (oval) occurred in single individuals of laboratory-reared colonies M and P (6×10^3 CFUs per whole gut in one termite of wood-fed colony M; 20 and 9×10^3 CFUs per whole gut in two filter paper-fed individuals of colony P). Coccobacillary only occurred in laboratory-reared individuals of colony M (39 and 8×10^3 CFUs per whole gut in two wood-fed individuals and 5×10^3 CFUs per whole gut in one filter paper-fed individual of the same colony). The latter three minor isolates seem to be transient flora, because they were only infrequently found in single termite workers. Therefore, they were not included in the statistical analysis.

General Linear model analysis showed a highly significant overall effect of the three rearing conditions combined ($P = 0.001$, Wilks' Lambda = 0.457, $F = 3.950$, df = 8), yet only a marginal effect of colony membership of the termite host ($P = 0.058$, Wilks' Lambda = 0.646, $F = 2.014$, df = 8) on the proportions of the four selected bacterial morphotypes in the termite guts (isolates 1–3, Enterobacteriacea, Fig. 2). Tests of between-subject effects revealed that the marginal colony effect was because of the proportions of the Enterobacteriacea

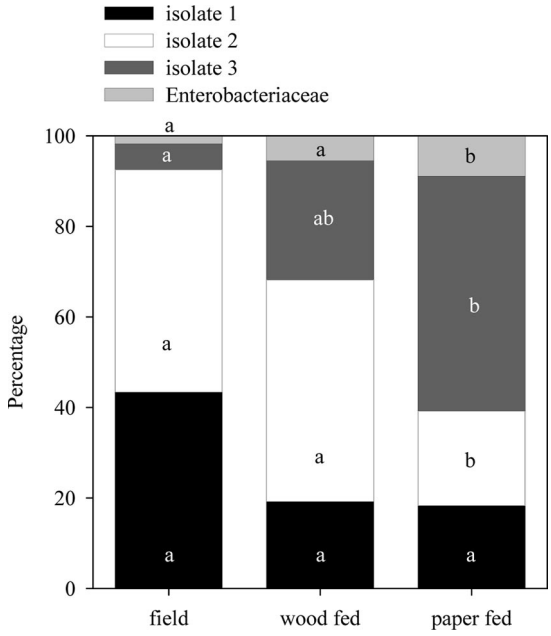

Fig. 2. Shift in the proportions of four morphotypes (isolates 1–3, Enterobacteriaceae) from freshly collected termites to termites reared in the laboratory in arenas on wood and in petri dishes on filter paper. Results are averaged over three termite colonies. Different letters indicate significant difference between the proportions of each morphotype in the three different rearing groups; note that letters indicate significances only between respective morphotypes from different rearing groups, not within rearing groups.

($F = 3.195$, df = 2; $P = 0.053$), whereas the rearing effect was because of changes in proportions of isolate 2, 3, and the Enterobacteriaceae (isolate 2: $F = 7.926$, df = 2, $P = 0.001$; isolate 3: $F = 4.225$, df = 2, $P = 0.022$; Enterobacteriaceae: $F = 7.743$, df = 2, $P = 0.002$). For a detailed post hoc analysis of the rearing effects, the three colonies were combined.

Separate post hoc tests showed that there were significant differences in the proportions of isolate 2, isolate 3, and the Enterobacteriaceae between termites freshly collected from their colony and those reared in the laboratory for 1 wk on filter paper (Tamhane's T2, $P < 0.050$ in each case). However, no significant difference could be detected in the proportions of these morphotypes between freshly collected termites and termites reared under seminatural conditions in the laboratory on wood (Tamhane's T2, $P > 0.20$ for isolate 2 and Enterobacteriaceae; $P = 0.094$ for isolate 3). Differences in the proportions of isolate 2 were significant between termites reared in the laboratory on wood versus filter paper (Tamhane's T2, $P = 0.025$), whereas differences in proportions of Enterobacteriaceae were marginal significant ($P = 0.089$). No significant difference was found in the proportions of isolate 3 between the two feeding conditions of the laboratory-reared termites ($P > 0.20$).

Discussion

Our study provides a general assessment of the culturable prokaryotic diversity in the gut of the major termite pest *C. formosanus* based on the use of relatively nonselective media and anaerobic conditions. Overall, we were able to culture, identify, and enumerate eight morphotypes recovered from the termite gut. Five major morphotypes were found in significant proportions in freshly collected termites of all three field colonies: isolate 1, isolate 2, and isolate 3 were three previously uncultured and unidentified species of the Lactobacillales. Although isolate 3 seems to be a novel species, closely related phylotypes of isolate 1 and 2 were found in a study of the gut flora of Japanese *C. formosanus* by using culture-independent 16S rDNA sequencing (Shinzato et al. 2005). Isolate 1 has since been described as the novel genus and species *Pilibacter termitis* (Higashiguchi et al. 2006). In addition to these three major isolates, two of the minor isolates were also species of lactic acid bacteria (coccobacillary and oval). Lactic acid bacteria colonize a diverse range of termite hosts with different habitats and diets, including subtropical and temperate regions as well as in soil and wood feeding species. Tholen et al. (1997) found lactic acid bacteria to comprise the dominant culturable bacteria population in *Reticulitermes flavipes* (Kollar). Eutick et al. (1978a,b) found various *Streptococcus* species of lactic acid bacteria as the dominant recoverable isolates in *M. darwinensis* and *Cryptotermes primus* Hill and as secondary isolates in *Heterotermes ferox* Froggatt, *Coptotermes lacteus* Froggatt, and *Nasutitermes exitiosus* Hill. Finally, Bauer et al. (2000) reported that lactic acid bacteria comprised the majority of all culturable isolates in *N. arborum* Noirot, *Thoracotermes macrothrorax* Sjöstedt, and *Anoplotermes pacificus* Mueller.

Lactic acid-producing bacteria are believed to play important roles in the gut ecology and nutrition of termites. For example, lactic acid bacteria maintain microoxic zones in the gut, which are important for the survival of strictly anaerobe microorganisms such as the protists, by scavenging oxygen. Lactic acid bacteria maintain homoeostasis in the gut via their metabolites and prevent invasion of the gut by opportunistic bacteria (Veivers et al. 1982, Bauer et al. 2000). Potrikus and Breznak (1980, 1981) demonstrated that several bacteria including lactic acid bacteria (e.g., *Streptococcus* spp.) isolated from *R. flavipes* were capable of recycling carbon and nitrogen by metabolizing uric acid. Because termites exist on a nitrogen poor diet, the assimilation of nitrogen back from uric acid would be a major benefit to the termites.

Members of the Enterobacteriaceae are generally found in many environments, including guts of other termite and insect species (Dillon and Dillon 2004). In this study, we were able to culture *Enterobacter cloacae* from termite guts and two strains that could not be identified using classical methods. In other Hawaiian termite colonies surveyed previously, we found *Citrobacter amalonaticus*, an unknown *Citrobacter* sp., *Klebsiella pneumoniae*, and *Kluyvera* sp. in addition to

Enterobacter cloacae and the two unknown strains described in this study (Husseneder et al. 2005).

Although satellite bacteria were found occasionally without neighboring Enterobacteriaceae colonies, the presence of Enterobacteriaceae increased the chance of finding this morphotype. This suggests that Enterobacteriaceae provide a metabolic byproduct that aids in the growth of satellite bacteria. These satellite bacteria were two *Dysgonomonas* species as putatively identified by 16S rRNA gene sequence analysis. The *Dysgonomonas* spp. belong to the order Bacteroidales, a bacteria order frequently detected in guts of subterranean termites (*Reticulitermes:* Fisher et al. 2007; *Coptotermes:* Husseneder et al. 2005, Shinzato et al. 2005). *Dysgonomonas* spp. isolated from the gut of the Formosan subterranean termite were able to produce vitamin B_{12}, an important supplement to the nutrition of the termite host (unpublished data).

Our study showed that there are only minor differences in the predominant culturable bacteria community among different termite colonies but that shifts in the termite gut flora can be induced by different rearing conditions and sources of nutrition. The variation in the proportions of the four morphotypes (isolates 1–3, Enterobacteriaceae) was comparatively low among the three termite colonies. The main contribution to intercolonial variance came from the group of Enterobacteriaceae. A possible explanation for this variance is that Enterobacteriaceae are facultative aerobic and are frequently found in the environment of the termites; bacteria could be picked up during foraging activity (Lee et al. 2009).

The lack of significant differentiation in the predominant gut flora among colonies was surprising. Because there is no behavioral interaction and thus no exchange of microbes between termites from different colonies, microbial populations are isolated from each other (Thorne 1997). Because of the rapid reproduction of bacteria and the ability of the microbial community to rapidly respond to selective pressures (Fernandez et al. 1999, 2000), we would have expected to find distinct bacterial compositions in different termite colonies. Furthermore, it has been suggested that aggression between termites from different colonies may be caused by differences in the bacterial composition among colonies (Matsuura 2001).

The lack of significant differentiation between the compositions of the microbial community of different colonies may be because of the limited number of individuals within colonies we surveyed and the reliance on cultured bacteria. However, other studies, including recent ones using high resolution molecular techniques, suggest that selective pressures balance the composition of the microbial flora; closely related termites harbor closely related gut bacteria (Hongoh et al. 2005, Yang et al. 2005) and termites of the same species possess similar microbial communities when living under similar environmental conditions even when collected from different locations, which suggests that selection in the hindgut favors a certain composition of microbes (Krasil'Nikov and Satdykov 1970, Thayer 1976, Eutick et al. 1978a, Schultz and Breznak 1978). Because of the vital role some bacteria play in the survival of termites, selective pressures should ensure that the most important microbe groups are always present. For example, isolate 1 (*P. termitis*, Higashiguchi et al. 2006) was present in all colonies; even under different rearing conditions, there was no significant shift. Interestingly, a very similar, if not identical bacteria species also was found among the ribotypes that were described by 16S rRNA gene sequencing of guts of Formosan subterranean termites from Japan (Shinzato et al. 2005), Louisiana (Husseneder et al. 2005), and China (unpublished data). The occurrence of this bacteria species in all examined termite guts across a wide geographic range suggests that *P. termitis* is an obligate symbiont of the Formosan subterranean termite that may play an important role in the ecological balance of the termite gut and/or for termite nutrition.

In contrast to the largely comparable bacterial species composition in different termite colonies, we found changes in the proportions of three bacterial morphotypes occurring within seven days after freshly collected termites were reared in the laboratory on filter paper. Rapid shifts in the microbial flora of the termite gut in response to drastic changes in rearing conditions have been documented previously. For example, removing termites from their colony, and the disturbance created by laboratory rearing, changed nitrogen-fixing rates in the termite gut (Breznak 2000). Breznak proposed that the gut microenvironment changed rapidly after removal of the termites from the colonies, because of increases in the ammonia concentration and/or the dissolved oxygen content, both of which inhibit nitrogenase activity. Another example of the effect of rearing termites in captivity is the accumulation of uric acid in *R. flavipes* and *N. walkeri* because of an increase in biosynthesis (Potrikus and Breznak 1980, Chappell and Slaytor 1993).

Martin and Kukor (1984) have shown that when termites are deprived of natural food sources they are likely to digest parts of their gut flora to assimilate proteinaceous food, lipids, carbohydrates, vitamins among others. Under natural conditions lost gut flora can be reinoculated by colony members, but this is prevented by laboratory rearing in small isolated groups. However, the termites in our study that were reared in the laboratory under seminatural conditions in arenas on wood contained similar proportions of bacterial morphotypes as field-collected termites. Thus, removal from the colony and rearing the termites in small groups in the laboratory were not the main causes of the shift. The nutrition of the termite seems to be the proximate cause of the shifting microbial flora.

Changes in the nutrient intake may create different selective pressures that favor different microbial composition while still preserving functional stability (Fernandez et al. 1999, 2000). Shifts in the composition of the gut flora depending on nutrition reflect the plasticity, adaptation, and competition

within the microbial community in termite guts. Gut microbes can adapt rapidly to changes in the insect diet by induction of enzymes and changes in species numbers and composition (Kaufman and Klug 1991, Dillon and Dillon 2004). For example, when cockroaches were fed on a low protein and high fiber diet the numbers of streptococci and lactobacilli decreased in the foregut (Kane and Breznak 1991). High cellulose content increased the protozoa population (Gijzen et al. 1994). When crickets were fed on cricket chow the dominant gut flora had a high G + C content, whereas pulp or protein diet increased the proportion of bacteria with low G + C content (Santo Domingo et al. 1998). When *C. formosanus* workers from the same laboratory colony were divided into two groups, one group fed with high-molecular-weight carbon sources and the other group with low-molecular-weight carbon sources, their bacterial gut community diverged to a point where 60% of the species were different (Tanaka et al. 2006). Change in the microbial flora because of availability of certain diet components may subsequently change living conditions in the gut, such as the pH, the redox potential, and the availability of metabolic products used by other microbes. This might shift the composition of the microbial flora permanently.

The reasons for rapid changes in the composition and activity of the gut flora in termites are not well understood. Mannesmann (1972) suggested that chemical compounds influence growth rate of microorganisms differently; as it has been shown for the protozoa species, physiological requirements likely differ among bacteria groups. We further need to investigate what roles the bacteria that are affected by different rearing conditions play in the ecological balance of the termite gut, what nutrition source they use, and what they provide for other microorganisms and the termite hosts themselves. The major part of this microbial diversity in termite guts is not yet represented in culture due to the inherent difficulties of culturing highly specialized, fastidious strains, which are refractory to isolation. Although culture-independent descriptions of the microbial community of *C. formosanus* are greatly contributing to the description of bacterial inventories (Husseneder et al. 2005, Shinzato et al. 2005, Fisher et al. 2007), culture is still necessary to test bacterial strains for their physiological and biochemical contribution to termite nutrition. Future research should shed light on the dynamics, functionality and adaptability of the intricate ecosystem of the termite gut and how this is linked to fitness of the individual host and the termite colony.

In conclusion, the microorganisms of the termite gut interact in a network, and together support the host and each other (Breznak 2000). We demonstrated rapid changes of this consortium depending on the diet of the host termites. Caution is advised when describing the "natural" gut flora of termites and other insects, because numbers and proportions of microbes change rapidly in response to rearing conditions.

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